

# PATENT COOPERATION TREATY

**PCT**

## NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

Assistant Commissioner for Patents  
United States Patent and Trademark  
Office  
Box PCT  
Washington, D.C.20231  
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

<b>Date of mailing (day/month/year)</b> 02 March 2000 (02.03.00)	
<b>International application No.</b> PCT/US99/16338	<b>Applicant's or agent's file reference</b> 5405.214.WO
<b>International filing date (day/month/year)</b> 19 July 1999 (19.07.99)	<b>Priority date (day/month/year)</b> 21 July 1998 (21.07.98)
<b>Applicant</b> VITEK, Michael, P.	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:

16 December 1999 (16.12.99)

☐ in a notice effecting later election filed with the International Bureau on:

2. The election ☒ was

☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

<b>The International Bureau of WIPO</b> 34, chemin des Colombettes 1211 Geneva 20, Switzerland	<b>Authorized officer</b>  S. Mafla
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38



## PATENT COOPERATION TREATY

## PCT

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

REC'D 09 OCT 2000

WIPO

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Applicant's or agent's file reference 5405.214.WO	FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/US99/16338	International filing date (day/month/year) 19 JULY 1999	Priority date (day/month/year) 21 JULY 1998
International Patent Classification (IPC) or national classification and IPC IPC(7): A01K 67/00; G01N 33/00 and US Cl.: 800/8, 3		
Applicant DUKE UNIVERSITY		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.


2. This REPORT consists of a total of 5 sheets.

☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 2 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☒ Certain observations on the international application

Date of submission of the demand 16 DECEMBER 1999	Date of completion of this report 15 SEPTEMBER 2000
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer  RAM R. SHUKLA
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196



## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US99/16338

## I. Basis of the report

## 1. With regard to the elements of the international application:\*

☒ the international application as originally filed☒ the description:

pages 1-12, as originally filed  
pages NONE, filed with the demand  
pages NONE, filed with the letter of

☒ the claims:

pages 13-14, as originally filed  
pages NONE, as amended (together with any statement) under Article 19  
pages NONE, filed with the demand  
pages NONE, filed with the letter of

☒ the drawings:

pages NONE, as originally filed  
pages NONE, filed with the demand  
pages NONE, filed with the letter of

☒ the sequence listing part of the description:

pages NONE, as originally filed  
pages NONE, filed with the demand  
pages NONE, filed with the letter of

## 2. With regard to the language, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language \_\_\_\_\_ which is:

- ☐ the language of a translation furnished for the purposes of international search (under Rule 23.1(b)).  
☐ the language of publication of the international application (under Rule 48.3(b)).  
☐ the language of the translation furnished for the purposes of international preliminary examination (under Rules 55.2 and/or 55.3).

## 3. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in printed form.  
☐ filed together with the international application in computer readable form.  
☐ furnished subsequently to this Authority in written form.  
☐ furnished subsequently to this Authority in computer readable form.  
☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.  
☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

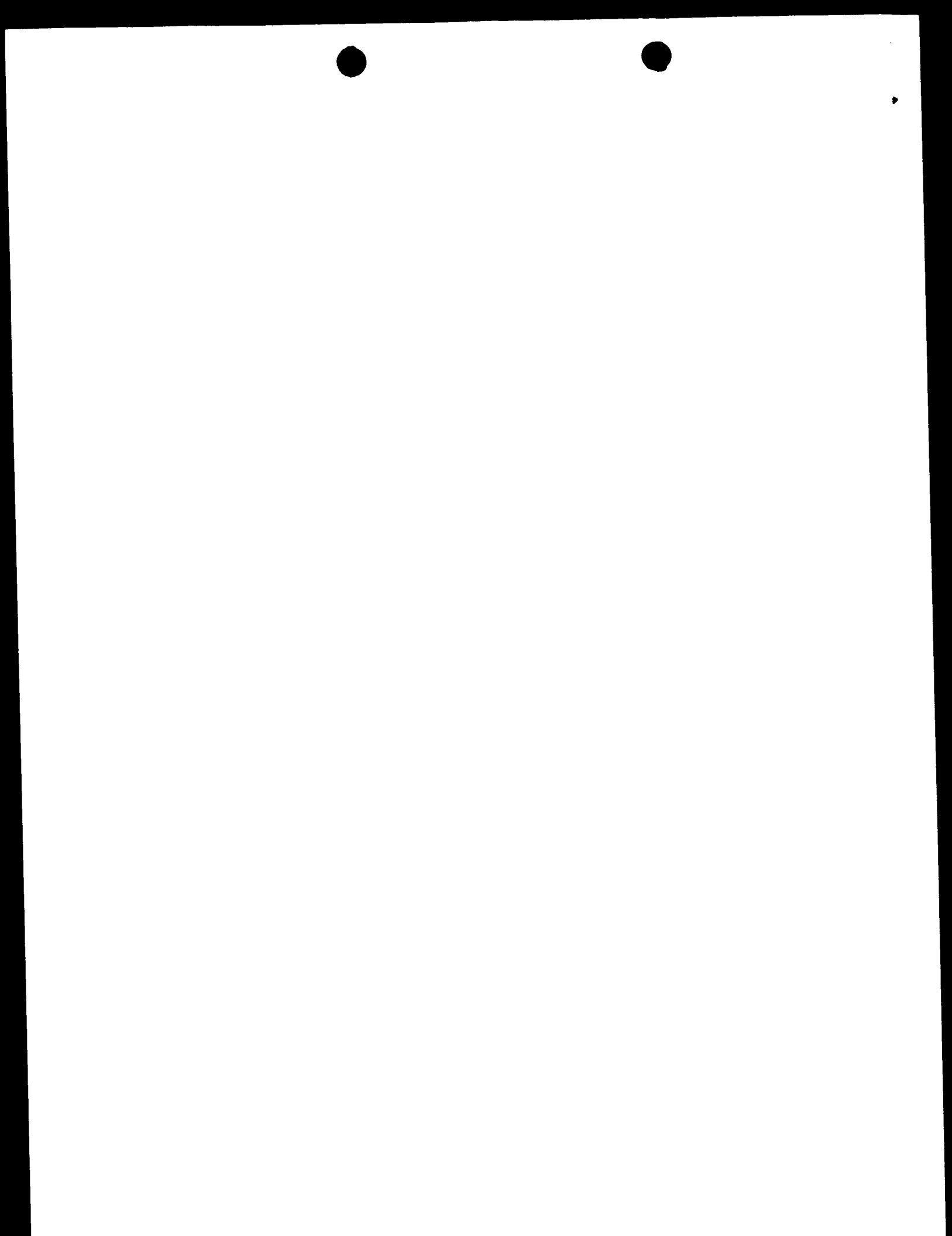
4. ☒ The amendments have resulted in the cancellation of:

☒ the description, pages NONE  
☒ the claims, Nos. NONE  
☒ the drawings, sheets/fig. NONE

5. ☒ This report has been drawn as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).\*\*

\* Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17).

\*\*Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.



## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US99/16338

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

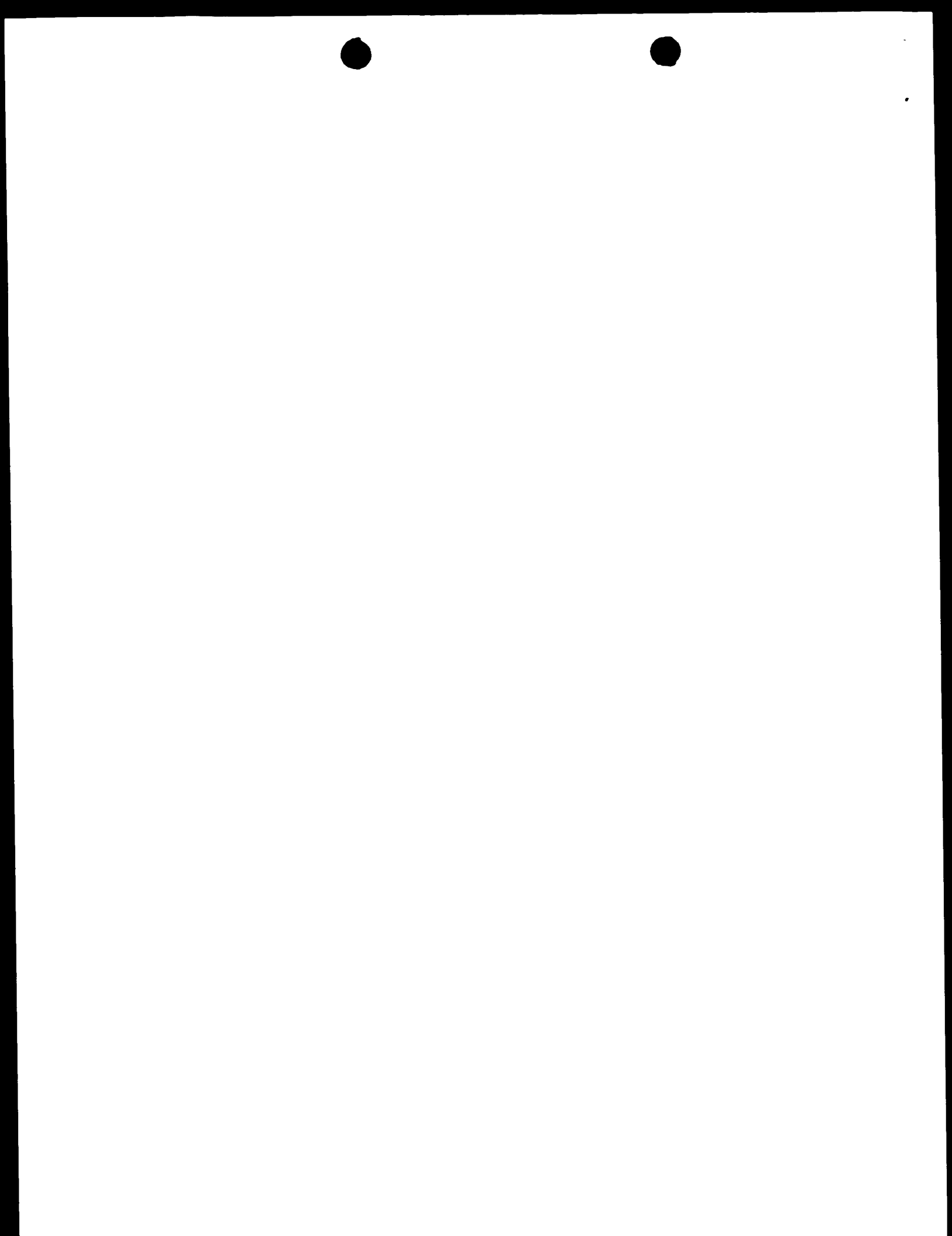
## 1. statement

Novelty (N)	Claims <u>1-10</u>	YES
	Claims <u>NONE</u>	NO
Inventive Step (IS)	Claims <u>1-10</u>	YES
	Claims <u>NONE</u>	NO
Industrial Applicability (IA)	Claims <u>1-10</u>	YES
	Claims <u>NONE</u>	NO

## 2. citations and explanations (Rule 70.7)

Claims 1-10 meet the criteria set out in PCT Article 33(2)-(4), because the prior art does not teach or fairly suggest: a transgenic mouse wherein the endogenous mouse inducible nitric oxide synthase gene has been inactivated and the germ and somatic cells of the mouse contain the human inducible nitric oxide synthase gene and a use of the mouse for screening compounds that alter the inducible nitric oxide synthase activity.

----- NEW CITATIONS -----  
NONE





INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

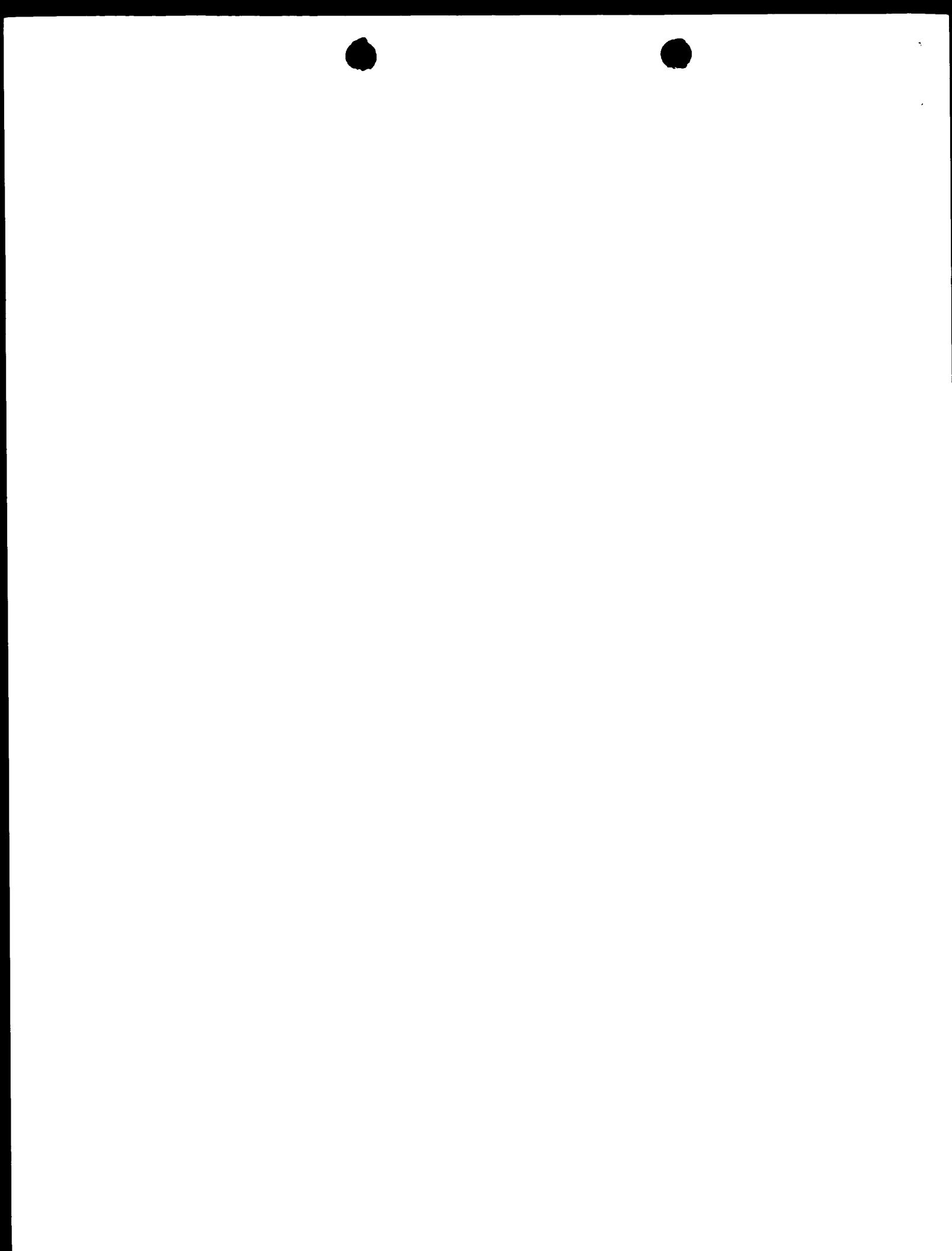
PCT/US99/16338

**VIII. Certain observations on the international application**

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

The description is objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 5 because it fails to adequately enable practice of the claimed invention because: The invention is to a transgenic mouse and to methods for screening compounds that induce or inhibit condition of immunopathological conditions, such as Alzheimer's disease. However, the description only provides prophetic example of making a transgenic mouse. While making of a transgenic mouse may be enabled, an artisan would not have known what were the phenotypes or characteristics of the transgenic mouse that would differentiate from the wild type mouse. Furthermore, an artisan would not have been able to know the phenotype of the transgenic animal because the murine gene of inducible nitric oxide synthase is inactivated and a human gene for the same is introduced. In the absence of the phenotypes, an artisan would not have known for what purpose and how to use the transgenic mouse in the claimed methods.

Claims 1-10 objected to as lacking clarity under PCT Rule 66.2(a)(v) because practice of the claimed invention is not enabled as required under PCT Rule 5.1(a) for the reasons set forth in the immediately preceding paragraph.



INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US99/16338

**Supplemental Box**

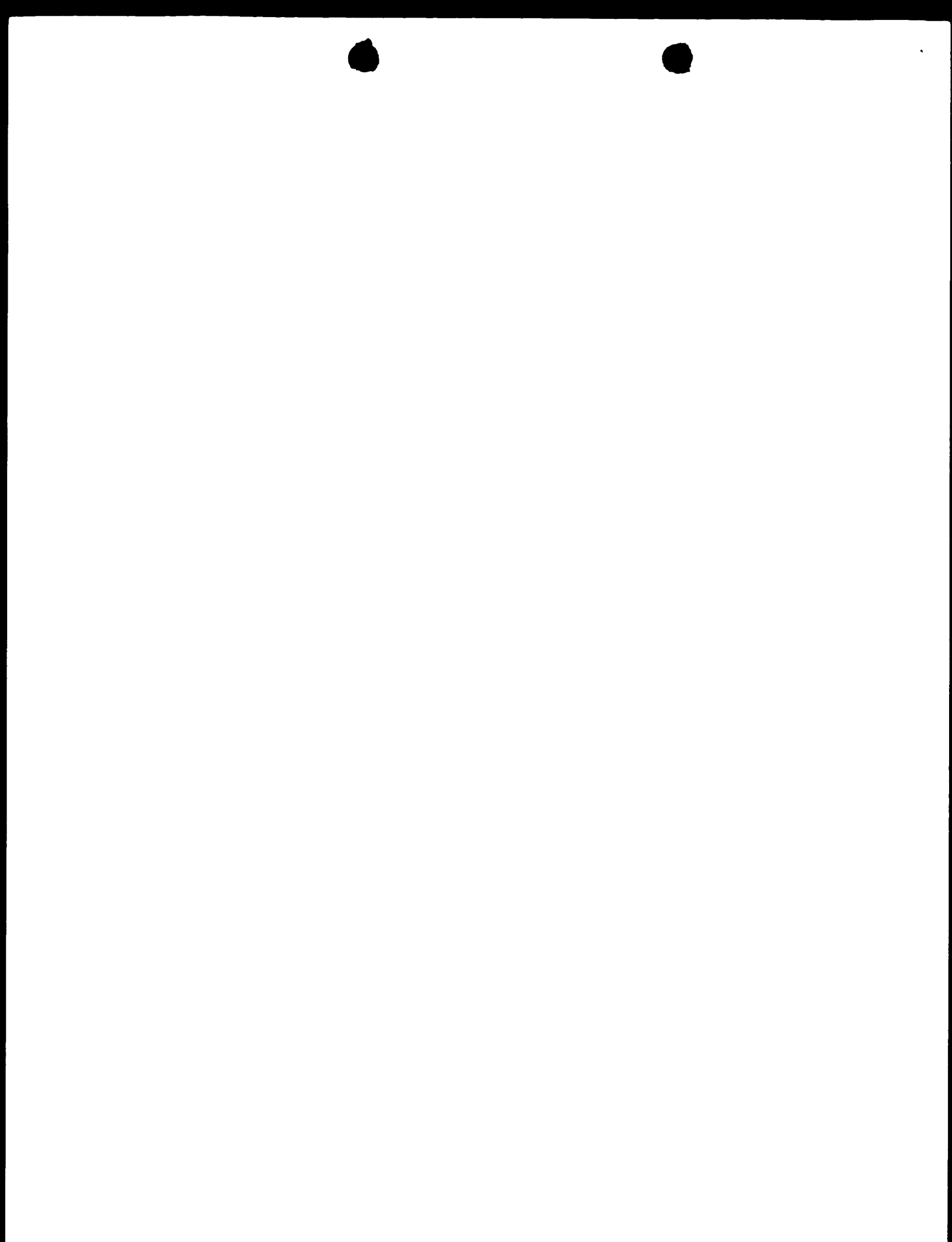
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

Sheet 10

**I. BASIS OF REPORT:**

5. (Some) amendments are considered to go beyond the disclosure as filed:  
NONE



**TRANSGENIC MODEL OF HUMAN OXIDATIVE STRESS**

Michael P. Vitek

This invention was made with Government support under grant numbers 1RO1 AG 13839 and 1RO3 AG 15609 from the National Institutes of Health. The Government has certain rights to this invention.

5

**Field of the Invention**

The present invention concerns transgenic animals, particularly transgenic mice, that are useful as models of human oxidative stress.

**Background of the Invention**

10 Nitric oxide is a molecule associated with human diseases of the brain and of peripheral tissues. Pathological roles for nitric oxide include cytotoxic and cytostatic effects upon bacterial invaders and toward cancer cells. The physiological roles for nitric oxide (NO) as an intra-cellular and inter-cellular messenger are the focus of intense investigation. Depending upon the situation tested, the co-localization of  
15 nitric oxide with diseased and dying cells, particularly in inflammatory conditions, suggest that NO plays an active role in protecting healthy cells from oxidative challenge or alternatively, directly participates in cellular degeneration.

Three different Nitric Oxide Synthase enzymes (NOS) convert arginine into citrulline and nitric oxide. The eNOS form associated with endothelial cells (NOS3  
20 gene) and the nNOS form associated with neuronal cells (NOS1 gene), appear to constitutively produce low levels of nitric oxide. In contrast, phagocytic cells like macrophages and microglia contain an immunological form known as iNOS (also known as inducible NOS)(NOS2 gene) which can be induced to release large amounts of nitric oxide. iNOS activity is highly regulated at the level of transcription. To  
25 further complicate this picture, iNOS activity is regulated in a species specific manner

such that treatment with an inducer like lipopolysaccharide, stimulates large increases in mouse-iNOS activity as measured by nitric oxide release, with virtually no effect on human-iNOS activity (Colton et al., 1996). In addition to species specific stimulation, mice display a high-output nitric oxide release system while human macrophages, even when maximally stimulated, display a low-output nitric oxide release system (Ding et al., 1997; Weinberg et al., 1995). In comparison, the amounts of nitric oxide produced by eNOS and nNOS are low and appear to be about the same in mice and humans (Wink, 1997). Thus, the use of mice in models of human inflammatory diseases, where nitric oxide is thought to play a critical role, should be rigorously questioned on the grounds that iNOS activity differs greatly in mice and men (or women).

In view of the foregoing, there is a need for a mouse model of human iNOS function that would be useful as a model of human oxidative stress.

15 **Summary of the Invention**

Disclosed is a transgenic mouse whose germ cells and somatic cells contain (i) an inactive mouse inducible nitric oxide synthase gene, and (ii) a transgene encoding the human inducible nitric oxide synthase gene, with the transgene including all regulatory elements of the human inducible nitric oxide synthase gene necessary for human patterns of expression of said transgene in said transgenic mouse. The mice of the invention may contain one or two alleles for the human inducible nitric oxide synthase gene (i.e., one or two NOS2 alleles).

Mice of the invention are useful as models of human inflammatory disease, including but not limited to Alzheimer's disease, Multiple Sclerosis, Inflammatory Bowel Disease, and Rheumatoid Arthritis. Thus, the ability of a compound to induce such diseases may be determined or screened by administering a test compound to an animal of the invention and then monitoring the animal for the development of the disease (*e.g.*, by monitoring for one or more sign, symptom or indicia of such a disease such as an underlying physiological event correlated to the disease). Further, the ability of a compound to treat such diseases may be determined or screened by administering a test compound to an animal of the invention and then monitoring that animal for treatment of the disease (*e.g.*, the alleviation, reduction, arresting or slowing of the progress of one or more sign, symptom or indicia of such a disease).

The present invention is explained in greater detail in the specification set forth below.

### Detailed Description of the Invention

5       The human NOS2 gene is known and can be obtained in accordance with known techniques. *See, e.g.*, Spitsin, S. et al., *Mol. Med.* **2**, 226-235 (1996); Vitek, M. et al., *BBRC* **240**, 391-394 (1997).

      Mice containing an inactive or inactivated mouse inducible nitric oxide synthase gene (e.g., knockout mice) are known and available from commercial  
10       sources (e.g., Jackson Labs, Bar Harbor, Maine, USA).

      The production of transgenic mice can be carried out in view of the disclosure provided herein and in light of techniques known to those skilled in the art, such as described in U.S. Patents Nos. 5,767,337 to Roses et al.; 5,569,827 to Kessous-Elbaz et al.; and 5,569,824 to Donehower et al. (the disclosures of which applicants  
15       specifically intend to be incorporated by reference herein in their entirety).

      By "human pattern of expression" is meant that the transgene is expressed in mouse phagocytic cells, particularly activated macrophages and/or activated microglia, and more particularly is meant that lipopolysaccharide (LPS) inducers induce essentially no increase in iNOS activity in phagocytic cells of a mouse of the  
20       invention as measured by nitric oxide release as compared to the corresponding wild-type mouse, and the phagocytic cells display low-output nitric oxide release in a mouse of the invention as compared to the corresponding wild-type mouse even when maximally stimulated. In one specific example, a mouse pattern of expression in elicited peritoneal macrophages from the mouse would include stimulation of nitric  
25       oxide production by LPS or IFN-gamma, and a human pattern of expression would be a lack of nitric oxide production following stimulation with these agents.

      By "corresponding wild-type mouse" is meant a mouse that does not contain the said transgene, and does contain at least one active mouse inducible nitric oxide synthase gene (e.g., one or two mouse NOS2 alleles).

30       To demonstrate how human iNOS contributes to human inflammatory diseases, described herein are transgenic mice lacking the mouse NOS2 gene ( $\mu$ NOS2  $-/-$ ) and containing at least one copy of the human NOS2 gene ( $huNOS +/0$ ) which are called "Humanized-NOS2" transgenic mice. Specifically disclosed are

how to clone and characterize a PAC clone of human genomic DNA containing the entire 40 kbp of the human NOS2 gene and at least 15 kbp of its promoter-regulatory sequences, and the use of this PAC clone to create and characterize transgenic mice containing the human NOS2 gene. Transgenic mice containing the human NOS2  
5 gene are mated to mice lacking the mouse NOS2 gene (available from Jackson Labs) to generate a hybrid transgenic mouse expressing only human iNOS (NOS2) enzyme, i.e. a "Humanized-NOS2" transgenic mouse. These mice are characterized for the expression of human NOS2 gene products.

An object of the invention is to produce a transgenic mouse whose  
10 physiological regulation of iNOS activity mimics that of humans. iNOS activity is tested in the mice to confirm it is regulated in a human-specific or in a mouse-specific pattern in these "Humanized-NOS2" transgenic mice. When the human NOS2 promoter/reporter construct is placed into mouse cells, its activity appears to mimic the human specific pattern and not the mouse activity pattern (Mitsuda et al., 1997;  
15 Spitsin et al., 1996). As described herein, the "Humanized-NOS2" transgenic mice also display a human-specific pattern of activity. With such mice, the role of iNOS and its nitric oxide products can be better defined in terms of how humans respond to stimuli typically associated with inflammation and neurodegeneration.

To use the mice as models of human disease, mice are stressed by injecting  
20 with myelin fractions to generate Experimental Allergic Encephalomyelitis (EAE), which is a model of Multiple Sclerosis (MS) in humans, and compare the severity of disease in wild-type, NOS2-Knockout and "Humanized-NOS2" transgenic mice treated with and without various inhibitors of Nitric Oxide Synthase. Adjuvant Induced Arthritis (AIA), which is a model of rheumatoid arthritis in humans, is also  
25 tested in these mice in a similar fashion. As the balance between superoxide and nitric oxide can define pro-oxidant and anti-oxidant conditions, a human specific pattern of nitric oxide production may correctly predict the role of nitric oxide in human pathological conditions. These "humanized-NOS2" transgenic animals are thus useful as a model to test NOS inhibitors and drugs affecting the arginine cycle.

30 Virtually all successful screening efforts to find drugs that effectively improve human disease conditions require those drugs to be tested in an animal model. A physiologically-relevant model of human nitric oxide production is herein provided. These animals serve as useful models of nitric oxide production as it pertains to the



generation of oxidative stress, which is implicated in the inflammation and destruction of cells in a wide variety of human inflammatory diseases. An important focus for such study is to measure the pro-oxidant and anti-oxidant potentials of nitric oxide in mouse models of inflammatory disease. It is well known that nitric oxide can  
5 combine with superoxide to form the strong oxidant, peroxynitrite, which, for example, can attack tyrosines in proteins to form nitrotyrosine residues as can be detected with monoclonal antibodies (Beckman, 1996). Since nitric oxide is also a radical, it can combine with the peroxynitrite radical to generate a non-radical species with the net effect of inhibiting, for example, protein and lipid oxidation. Thus, drugs  
10 which modulate nitric oxide production can be tested in the "Humanized-NOS2" animals described herein for their pro-oxidant and/or anti-oxidant potentials, their ability to relieve pain and suffering associated with inflammation and cell destruction in tissues brought about by release of inflammatory modulators.

As an additional utility, the protein products of genes associated with  
15 Alzheimer's disease (AD) can stimulate nitric oxide production. Using primed human monocyte derived macrophages as models of microglia, apolipoprotein-E can stimulate nitric oxide production. These same cells fail to produce nitric oxide in response to treatment with Amyloid-beta peptide (Abeta). Since mouse cells do produce nitric oxide in response to Abeta treatment (Meda et al., 1995), mice differ  
20 from humans in their response to stimulation of nitric oxide production (see also Walker et al., 1995). Since studies of microglial-mediated production of nitric oxide in human brains are limited by tissue availability, this "Humanized-NOS2" transgenic mouse provides a convenient animal model in which to study nitric oxide production relevant to that found in humans under physiological and pathological conditions.

25 While the present invention has been explained primarily with reference to mice, it will be appreciated that the invention can be implemented with other mammalian species, such as rats, dogs, cats, and monkeys, in accordance with known techniques, or techniques that will be apparent to those skilled in the relevant arts.

The present invention is explained in greater detail in the following non-  
30 limiting examples.

**EXAMPLE 1*****Cloning and Characterizing the Human-NOS2 gene***

Several PAC clones of human genomic DNA are obtained that are positive for the Promoter/Exon-1 region and for the Exon-27 region of the human NOS2 gene.

- 5 Each clone that contained both the 5' and 3' ends of the human NOS2 gene, is subjected to additional PCR reactions with primer pairs to each of the 27 Exons. Each of these Exon-specific PCR products is sequenced and compared to the reported sequences. A restriction map of each of these clones is made and compared to the reported restriction enzyme map to also ensure that the PAC clone is correct.
- 10 Additional mapping employs Southern blots of the positive PAC clones and of human genomic DNA, digested with Hind III, which will be hybridized with Exon-specific DNA probes (Vitek et al., 1984; Xu et al., 1994). The PAC clone(s) containing all of the human Exons, with the correct DNA sequences and restriction maps matching human genomic DNA, are then used for generating the "human-NOS2" transgenic
- 15 mouse.

**EXAMPLE 2*****Making the "human-NOS2" Transgenic Mouse***

- The entire human-NOS2 gene is used to generate the "human-NOS2"
- 20 transgenic mouse by conventional methods with a service provider such as DNX (Princeton, New Jersey, USA). Briefly, DNA from a PAC clone that meets all of the criteria listed above for containing a human NOS2 gene, is used to generate transgenic mice. PAC clone #2 contains both the Promoter/Exon-1 region and the Exon-27 region of the human--NOS2 gene in about 100 kbp of human genomic DNA. PAC
- 25 clone DNA is purified according to the instructions provided by the provider. PAC clone #2 is linearized with Not I. Not I is reported not to cleave the human NOS2 gene and it does not cleave the human NOS2 gene in PAC clone #2 which has been isolated, but Not I does cleave at both ends of the PAC vector as confirmed by pulse-field agarose gel electrophoresis. Linearized DNA is then injected into the pronucleus
- 30 of a fertilized mouse egg by the service provider. The injected eggs are reimplanted into pseudopregnant female mice who then give birth to the babies, the putative F<sub>0</sub> founders.

Genomic DNA extracted from snips of the end of the tails (snips of typically 2 to 5 mm in length) of the fully weaned pups are used to obtain the genotypes of each animal including putative "human-NOS2" transgenic mice (Xu et al. 1996a). After about 3 weeks when the pups are weaned, tail snips are taken and identification tags placed on each animal's ears. Genomic DNA is then extracted from the tails of these  $F_0$  mice (putative founders). Tail snips are minced with a fresh razor blade, placed in 0.4 ml of TE (10mM Tris-HCl, pH 7.4 and 1 mM EDTA, pH 7.4), extracted twice with 2 volumes of phenol:chloroform:isoamyl alcohol (50:48:2), re-extracted with 2 volumes of chloroform:isoamyl alcohol (24:1) and re-extracted with 2 volumes of ether. Residual ether in the aqueous phase is removed by drying under a stream of nitrogen gas. This DNA is genotyped by PCR for the presence of the human NOS2 gene using human-specific DNA probes from human NOS2's Promoter/Exon-1 region (Forward Promoter Primer = CCTTTCCTTCCAAAAACCTC; Reverse Exon-1 Primer = TCACCCAACCCACCTCTTTC to give a 345 bp product). A second PCR from the mouse TAU gene is also performed as a control for the PCR technique and for the presence of mouse DNA using a forward primer (TAU Exon-14 Forward Primer = TTGGCACTTCGATGATGACCTC) and reverse primer (TAU Exon-14 Reverse Primer = CATTGTGACGTGTGATGAGGGG) which give a PCR product of 420 bp whose sequence matches that reported (Andreadis et al., 1992). Southern blots of genomic DNA digested with Hind III and hybridized with the pIN-2 probe (spanning positions +2401 to +4203 of the human NOS2 cDNA which corresponds to Exon-18 to Exon-27 of the human NOS2 gene), are also employed. In this Southern paradigm, human NOS2 gene gives multiple bands of 12, 9.5, 8, 6, 4.5, 4 and 3.6 kBp, while mouse genomic DNA gives only one band of 7.6 kBp (Xu et al. 1994). Hybrid mice containing the human NOS2 and the mouse NOS2 genes contain the mouse-specific 7.6 kBp band and all 7 of the human-specific bands. In an alternative Southern paradigm, BamHI digest of genomic DNA probed with oligonucleotides from the sequence for Exon-18 are predicted to give a 14 kBp band from wild-type mouse DNA, a 10 kBp band from mu-NOS2-knockout DNA and a 2 kBp band from human DNA (Laubach et al., 1995; Xu et al. 1996b). Non-transgenics have a 14 kBp band hybridizing to an Exon-18 probe, while the hemizygous "human NOS2" transgenic mouse display bands at 14 kBp and at 2 kBp hybridizing to the Exon-18 probe.

F<sub>0</sub> mice containing the human NOS2 gene are mated to wild-type mice and their offspring (F<sub>1</sub> generation), ear tagged and genotyped for the presence of the human-NOS2 gene from tail snip DNAs as described above. F<sub>1</sub> mice carrying the human NOS2 gene have passed this gene through the germ line and are hemizygous.

5 These mice are then be used for matings as described below.

### EXAMPLE 3

#### *Breeding and Identifying a "Humanized-NOS2" Transgenic Mouse*

Mice hemizygous for the human NOS2 gene (generated as described above)

10 are mated to NOS2 knockout mice to generate a transgenic mouse expressing only human NOS2 gene products. The simplest way to generate such a mouse is to mate the hemizygous human NOS2 mice (which are +/0 with respect to the human NOS2 gene and +/+ with respect to the murine NOS2 gene) with homozygous NOS2 knockout mice (which are -/- with respect to the murine NOS2 gene and lack

15 functional muNOS2 genes). The resulting F1 hybrids of this first cross display an "H-Mm" genotype (arbitrary letter designation) which is +/0 for the huNOS2 gene and +/- for the muNOS2 gene and a "Mm" genotype which is -/- for huNOS2 and +/- for muNOS2. Equal numbers of the "H-Mm" and "Mm" genotypes are observed in this cross. Mice of the "H-Mm" genotype are then mated again to the muNOS2 knockout

20 mice (homozygous -/- for functional muNOS2) to generate F2 hybrid mice that display a "H-mm" genotype which is +/0 for huNOS2 gene and -/- for muNOS2 gene and a "Mm" genotype which is -/- for huNOS2 and +/- for muNOS2. Equal numbers of the "H-mm" and the "Mm" genotypes are observed in this cross. The "H-mm" genotype mice are then characterized for the expression of only human NOS2 gene

25 products and the nitric oxide produced from the human NOS2 gene products is found to be expressed in a human specific pattern.

Several methods are available to confirm the genotype of the transgenic mice.

A PCR strategy with a promoter/Exon-1 primer pair gives a specific band from the human NOS2 transgene DNA of about 477 bp. Also, as an alternative Southern

30 paradigm, BamH1 digests of genomic DNA probed with oligonucleotides from the sequence of Exon-18 are predicted to give a 14 kbp band from wild-type mouse DNA, a 10 kbp band from muNOS2 knockout DNA and a 2 kbp band from human DNA (Laubach et al, 1995; Xu et al 1996b). "H-Mm" mice display Exon-18

hybridizing bands at 2 kBp (from huNOS2), 14 kBp (from muNOS2) and 10 kBp (from the disrupted muNOS2 gene of knockout mice). "Mm" mice display Exon-18 hybridizing bands at 14 kBp and at 10 kBp. "H-mm" mice display Exon-18 hybridizing bands at 2 kBp and at 10 kBp. In this way, one can use Southern blots of  
5 tail snip DNA to diagnose the genotype of each of the transgenic hybrid mouse strains. As another alternative, one can perform test matings by crossing "H-mm" mice to "mm" muNOS2 knockout mice which will give half of the offspring carrying the human NOS2 transgene and the other half lacking it.

10

#### EXAMPLE 4

##### *Characterizing Human-NOS2 Gene Products*

Human NOS2 transgene expression is assessed at the RNA and at the protein levels. To measure human NOS2-mRNA, RNA is extracted from brain, denatured and Northern-blot the RNA, hybridize to the human-specific NOS2 probe and  
15 examined for bands of about 4.2 kB in size as reported by Charles et al. (1993). Although mouse NOS2 mRNA has been reported to also run in the 4 kB range (Laubach et al. 1995), it should not cross-hybridize to our probe which derives from human NOS2 Exons-1, -2 and -3 which are specific for the human-NOS2 sequence and do not share homology with the mouse NOS2 gene or with any non-human-NOS2  
20 sequence reported in Genbank. As a positive control for hybridization, probe with an APP cDNA for bands in the 3 kB range (Vitek et al. 1988). If the human-NOS2 transgene is being transcribed and post-transcriptionally spliced in mice, in a manner similar to that found in humans, then animals carrying the human-NOS2 transgene express RNA transcripts that hybridize to the human-specific NOS2 probe while wild-  
25 type mice, lacking the transgene, will not display hybridizing transcripts as visualized on Northern blots.

The human iNOS protein and the mouse iNOS protein share high homology at the amino acid sequence level. Although several commercially available antibodies recognize 130 kDa bands on Western blots, the inability of these antibodies to  
30 discriminate human from mouse proteins leads us to propose this method only if Northern blots fail to provide the desired information. Thus, a combination of DNA-genotype and bands on a Western could be used to diagnose the presence of human iNOS protein product of the human NOS2 transgene.

**EXAMPLE 5***Uses of Humanized-NOS2 Mice*

A "Humanized-NOS2" transgenic mouse expressing only human iNOS protein, is initially useful to test whether the regulation of human-NOS2 induction follows a human-specific or a mouse-specific pattern when in a mouse host. The induction pattern is measured following stimulation of nitric oxide synthase activity with various inducers by indirectly measuring nitrite as a marker of enzymatically-generated nitric oxide release. Since the levels of enzymatic activity of eNOS and nNOS are typically constitutive in peritoneal macrophages and represent a fraction of the inducible iNOS activity which they possess, then peritoneal macrophages are harvested from wild-type, muNOS2 knockout and from "Humanized-NOS2" transgenic mice for placement in culture and treatment with stimulating agents followed by nitrite (nitric oxide) measurement. Laubach et al. (1995) report that elicited peritoneal macrophages from wild-type mice, treated with lipopolysaccharide (LPS) and Interferon-gamma (IFN-gamma), have about 15 times more NOS activity than similarly treated cells of NOS2 knockout mice. MacMicking et al. report that nitrite levels in elicited peritoneal macrophages from wild-type mice treated with LPS and/or IFN-gamma, are at least 15 fold higher than similarly treated cells of a different NOS2 knockout mouse (MacMicking et al., 1995). Weinberg et al. (1995) report that human peritoneal macrophages produce little or no nitrite (ie. nitric oxide) before or after treatment with LPS, IFN-gamma or both. These data indicate that a mouse-specific pattern of nitric oxide production in elicited peritoneal macrophages would include stimulation by LPS or IFN-gamma and that a human-specific pattern would be a lack of nitric oxide production following stimulation with these agents. As suggested by Ding et al (1997), iNOS activity and nitrite release are increased following stimulation of human microglia, the brain-specific macrophage, with a combination of Interferon-gamma and Interleukin-1 beta (IL-1beta). This combination of IFN-gamma and IL-1beta are used as a positive control to show that the peritoneal macrophages from various mice are capable of showing an inducible rise in nitrite levels. Alternatively, one can culture microglia from the various mice and test their responses to these inducers.

Specific methods for obtaining peritoneal macrophages, measuring nitrite via Greiss reaction and culturing mouse brain microglia are found in Laubach et al. and

Colton et al. (Colton and Gilbert, 1993; Laubach et al., 1995). Briefly, macrophages are elicited by intraperitoneal (i.p.) injection of 2 ml of 4% Brewer thioglycollate (Difco). Four days following injection, cells are harvested by washing out the peritoneal cavity with 20 ml of cold phosphate buffered saline and the entire solution  
5 centrifuged to pellet the cells. Cells are resuspended in 20 ml of RPMI 1640 with 10% fetal bovine serum (RPMI-FBS) (Gibco-BRL-Life Technologies), centrifuged, cell pellets resuspended in RPMI-FBS and plated in 4 x 35 mm tissue culture dishes per mouse. After 3 hours at 37°C to allow cells to attach, non-adherent cells are removed with 3 washes of RPMI-FBS and adherent cells used for stimulation  
10 experiments. Adherent cells are treated with nothing (as a non-stimulated control), murine IFN-gamma (100 units/ml, Boehringer Mannheim) or LPS (1 µg/ml, E. coli 055:B5, Sigma) for 12, 24, 36, and 48 hours. At each time point, quadruplicate 50 µl aliquots of conditioned media are removed to 96 well tissue culture plates and nitrite levels assayed with the Greiss reaction. Briefly, an equal 50 µl aliquot of Greiss  
15 reagent (a 1:1 solution of 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride: 1% sulfanilamide in 5% phosphoric acid), incubated for 10 minutes at room temperature, and absorbance read at 544 nm of a plate reader. Sodium nitrite standards were employed to generate a standard curve of the colorimetric response. The method of Colton et al. is employed to prepare primary cultures of mouse brain microglia and  
20 test their responses to stimuli with the Greiss reaction listed above (Colton and Gilbert, 1993).

The foregoing is illustrative of the present invention, and is not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

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**That which is claimed is:**

1. A transgenic mouse whose germ cells and somatic cells contain (i) an inactive mouse inducible nitric oxide synthase gene, and (ii) a transgene encoding the human inducible nitric oxide synthase gene, said transgene including all regulatory elements of the human nitric oxide synthase gene necessary for a human pattern of expression of said transgene in said transgenic mouse.  
5
2. A transgenic mouse according to claim 1, wherein said mouse contains one allele encoding the human inducible nitric oxide synthase gene.  
10
3. A method of determining if a compound is capable of inducing Alzheimer's disease, comprising administering said compound to a mouse of claim 1, and then examining said mouse for one the development of said disease.
- 15 4. A method of determining if a compound is capable of inducing Multiple Sclerosis, comprising administering said compound to a mouse of claim 1, and then examining said mouse for the development of said disease.
- 20 5. A method of determining if a compound is capable of inducing Inflammatory Bowel Disease, comprising administering said compound to a mouse of claim 1, and then examining said mouse for the development of said disease.
- 25 6. A method of determining if a compound is capable of inducing Rheumatoid arthritis, comprising administering said compound to a mouse of claim 1, and then examining said mouse for the development of said disease.

7. A method of screening a compound for activity in treating Alzheimer's disease, comprising administering said compound to a mouse of claim 1, and then examining said mouse for the treatment of said disease.

5        8. A method of screening a compound for activity in treating Multiple Sclerosis, comprising administering said compound to a mouse of claim 1, and then examining said mouse for the treatment of said disease.

9. A method of screening a compound for activity in treating Inflammatory  
10 Bowel Disease, comprising administering said compound to a mouse of claim 1, and then examining said mouse for the treatment of said disease.

10. A method of screening a compound for activity in treating Rheumatoid  
arthritis, comprising administering said compound to a mouse of claim 1, and then  
15 examining said mouse for the treatment of said disease.

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US99/16338

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A01K 67/00; G01N 33/00

US CL : 800/8, 3

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/8, 3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, CAPLUS, WEST1.2

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P	EP 0 864 648 A1 (NUNOKAWA et al) 16 September 1998, see entire document.	1-10
Y, P	US 5,850,004 A (MACMICKING et al) 15 December 1998, see entire document.	1-10
Y	US 5,658,565 A (BILLIAR et al) 19 August 1997, see entire document.	1-10
Y	WOL et al. Altered immune responses in mice lacking inducible nitric oxide synthase. Nature. 01 June 1995, Vol. 375, pages 408-411, see entire document.	1-10



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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*P* document published prior to the international filing date but later than the priority date claimed	

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